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(71) Applicant *(for all designated States except US):* DUKE UNIVERSITY [US/US]; Erwin Road, Durham, NC 27706 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only) :* STRITTMATTER, Warren, J. [US/US]; 3817 Shippenham Road, Durham, NC 27707 (US).

(74) Agents: SIBLEY, Kenneth, D. et al.; Bell, Seltzer, Park & Gibson, P.O. Drawer 34009, Charlotte, NC 28234 (US).

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(54) Title: METHOD OF INHIBITING BINDING OF AMYLOID PRECURSOR PROTEIN TO BETA-AMYLOID PROTEIN

(57) Abstract

Methods of inhibiting the binding of β-amyloid protein to the amyloid precursor protein are disclosed, along with constructs comprising a β-amyloid protein or fragment thereof immobilized on a solid support, methods of detecting compounds which bind to β-amyloid protein, and methods of detecting compounds which inhibit the binding of the amyloid precursor protein to β-amyloid protein.

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METHOD OF INHIBITING BINDING OF AMYLOID PRECURSOR PROTEIN TO BETA-AMYLOID PROTEIN

This invention was made with Government support under NIH LEAD Award 5R35 AGO 7922 and NIH Alzheimer's Disease Research Center 5P5) AGO 5128. The Government has certain rights to this invention.

Related Applications

This application is a continuation in part of copending application Serial No. 07/959,251, the disclosure of which is incorporated by reference herein in its entirety.

Field of the Invention

The present invention relates to Alzheimer's disease, and particularly relates to senile plaque formation in Alzheimer's disease.

Background of the Invention

The senile plaque and congophilic angiopathy are abnormal extracellular structures found in abundance in brain of patients with Alzheimer's disease. The biochemical composition of these structures has been extensively studied to better understand their possible role in the pathogenesis of this dementing disease. The

mature senile plaque is a complex structure, consisting of a central core of amyloid fibrils surrounded by dystrophic neurites, axonal terminals and dendrites, microglia and fibrous astrocytes. *See* D. Selkoe *Neuron* **6**, 487-498 (1991). The amyloid core of the senile plaque and surrounding the blood vessels, producing the congophilic angiopathy, is a peptide of 39 to 43 amino acids termed the β-Amyloid (βA) peptide. G. Glenner and C. Wong, *Biochem. Biophys. Res. Comm.* **120**, 885-890 (1984). βA peptide is found in brain in Alzheimer's disease, Down's syndrome, hereditary cerebral hemmorhage of the Dutch type, and in old age. K. Kosik *Science* **256**, 780-783 (1992). βA is produced by abnormal proteolytic processing of a larger protein, the amyloid precursor protein (APP). *See* K. Beyreuther and C. Masters, *Brain Path.* **1**, 241-251 (1991).

The senile plaque and congophilic angiopathy contain proteins in addition to βA peptide. APP itself, among others, has been identified in the senile plaque by histochemical studies employing antibodies recognizing either the amino- and carboxy-termini of the precursor protein. *See, e.g.,* F. Tagliavine et al., *Neurosci. Lett.* **128**, 117-120 (1991); C. Joachim et al., *Amer. Jour. Path.* **138**, 373-384 (1991); The mechanisms by which these proteins aggregate in the extracellular space to associate with the senile plaque and congophilic angiopathy are not known.

P. Gorevic et al., *Biochem. and Biophys. Res. Comm.* **147**, 854 (1987) and E. Castano et al., *Biochem. and Biophys. Res. Comm.* **141**, 782 (1986) each describe a 12-28 amino acid domain of βA involved in βA self assembly into fibrils in the amyloid plaque of Alzheimer's disease patients. D. Kirschner et al., *Proc. Natl. Acad. Sci. USA* **84**, 6953 (1987) and D. Burdick et al., *J. Biol. Chem.* **267**, 546 (1992) each explore βA analogs. The synthetic peptide containing amino acids 12-28 of βA peptide (abbreviated $\beta A_{(12-28)}$) forms Congo red-staining fibrils 8-10 nm long. A

therapeutic use of these fragments and analogs is not suggested.

E. Kline et al., PCT Appln WO 91/16819 describes a method of treating Alzheimer's disease by administering the amyloid beta protein itself, or an active fragment thereof.

H. Potter, PCT Appln WO 92/03474, describes a therapeutic method of treating individuals, such as Alzheimer's disease patients, to prevent the formation of an α-antichymotrypsin - β-protein complex by administering to the subject a synthetic peptide comprising a fragment of the Alzheimer's amyloid β-protein, and particularly describes fragment 1-12 or 1-28.

While there has been considerable research into the mechanisms underlying Alzheimer's disease, there continues to be an ongoing need for new ways to investigate and combat this disorder.

Summary of the Invention

A first aspect of the present invention is a construct comprising a β-amyloid protein or fragment thereof (e.g., a fragment which binds to amyloid precursor protein) immobilized on a solid support.

A second aspect of the present invention is a method of detecting compounds which bind to β-amyloid protein. The method comprises providing a construct as given above, contacting an aqueous solution (e.g., cerebrospinal fluid) containing a compound suspected of binding to the β-amyloid protein to the construct; and detecting the presence or absence of target compound bound to said construct.

A third aspect of the present invention is a method of detecting compounds which inhibit the binding of amyloid precursor protein to β-amyloid protein. The method comprises providing an aqueous solution containing a binding pair, the binding pair comprising *(i)* the amyloid precursor protein or a fragment thereof which

binds to the β-amyloid protein, and *(ii)* the β-amyloid protein or a fragment thereof which binds to the amyloid precursor protein; adding a test compound to the aqueous solution; and then detecting whether or not the test compound inhibits binding between the members of said binding pair. In one embodiment, one member of the binding pair may be immobilized on a solid support.

A fourth aspect of the present invention is a method of inhibiting the binding of amyloid precursor protein to the β-amyloid protein. The method comprises contacting to one member of the amyloid precursor protein-β-amyloid protein binding pair a fragment of the other member of the binding pair, wherein said fragment binds to said one member, and wherein the fragment is provided in an amount sufficient to inhibit binding of amyloid precursor protein to β-amyloid protein. In one embodiment, the one member is β-amyloid protein and the fragment is an amyloid precursor protein fragment; in another embodiment, the one member is amyloid precursor protein and the fragment is a β-amyloid protein fragment.

A fifth aspect of the present invention is a method of detecting compounds which inhibit the binding of β-amyloid protein to Apolipoprotein E4. The method comprises, first, providing an aqueous solution containing a binding pair, the binding pair comprising *(i)* a first compound selected from the group consisting of Apolipoprotein E4 and fragments thereof which bind to the β-amyloid protein, and *(ii)* the β-amyloid protein or a fragment thereof which binds to Apolipoprotein E4, then, adding a test compound to said aqueous solution, and then, detecting whether or not the test compound inhibits binding between the members of the binding pair.

A sixth aspect of the present invention is a method of inhibiting the binding of Apolipoprotein E4 to β-amyloid protein. The method comprises contacting to one member of the Apolipoprotein E4-β-amyloid protein binding pair a fragment of the other member of the binding pair,

wherein the fragment binds to the one member, and wherein the fragment is provided in an amount sufficient to inhibit binding of Apolipoprotein E4 to β-amyloid protein.

The foregoing and other objects and aspects of the present invention are explained in detail in the drawings herein and the specification set forth below.

Brief Description of the Drawings

Figure 1 illustrates the binding of proteins in human cerebrospinal fluid to $\beta A_{(1-28)}$ peptide (SEQ ID NO:1) or to ethanolamine-immobilized to Immobilon AV membranes.

Figure 2 illustrates the binding of proteins in human cerebrospinal fluid proteins to $\beta A_{(12-28)}$ peptide (SEQ ID NO:2), or to the 12-28 Hydropathic Mimic Peptide (H.M.)(SEQ ID NO:3) immobilized to Immobilon AV membranes.

Figure 3 schematically illustrates the structure of various APP isoforms and APP deletion mutants.

Figure 4 illustrates the binding of recombinant-expressed human APP isoforms to $\beta A_{(1-28)}$ peptide or to ethanolamine immobilized to Immobilon AV.

Figure 5 illustrates the binding of 770 APP and deletion APP isoforms 770 KX$_\Delta$ and 751 KX$_\Delta$ to $\beta A_{(1-28)}$ peptide (βA) and to ethanolamine-control (C) immobilized Immobilon AV membranes.

Figure 6 shows the effect of reducing disulfide bonds, and of pH, on the binding of 695-K to $\beta A_{(1-28)}$ peptide.

Figure 7 shows the time course of SDS-stable binding of βA4 peptide by apoE3 and apoE4. One μl of apoE3 (A) or apoE4 (B) was incubated with $\beta A4_{(1-28)}$ (2.5×10^{-4}M) in a total volume of 20μl between 5 minutes and 24 hours at 37°C. The incubation was ended by adding 20 μl 2X Laemmli buffer (without B-mercaptoethanol) and boiling five minutes. Proteins were electrophoretically separated on a 7.5% polyacrylamide gel, and transferred to Immobilon P membrane. Both apoE and βA4 peptide were detected with the anti-apoE anitbody and the anit-βA4 peptide antibody.

Lane 1, apoE alone; Lane 2, βA4 peptide alone. Lanes 3-9 incubation of apoE with βA4 peptide for: Lane 3, 5 min.; Lane 4, 20 min; Lane 5,2 hrs; Lane 6,4 hrs; Lane 7,6 hrs; Lane 8, 12 hrs; Lane 9,24 hrs.

Figure 8 shows the effects of reducing agents β-mercaptoethanol or dithiothreitol on SDS-stable binding of βA4 by apoE. One μg apoE3 (A) or apoE4 (B) were incubated with $\beta A4_{(1-28)}$ (2.5×10^{-4}M) for five hours at 37°C. The incubation was stopped by the addition of an equal volume of 2X Laemmli buffer (without β-mercaptoethanol), and boiled five minutes. Lanes 1 and 4 contained apoE and βA4 peptide without reducing agents; Lane 2 with 0.2% (V/V) β-mercaptoethanol during the incubation; Lane 3 with 30 mM dithiothreitol during the incubation. In Lane 5, β-mercaptoethanol was added after the incubation, in Lane 6 dithiothreitol was added after the incubation. Proteins were electrophoresed on a 12% polyacrylamide gel, tansferred to Immobilon P membranes, and the βA4 peptide/apoE complexes were detected by anit-βA4 peptide antibody.

Figure 9 shows the effect of O_2 and N_2 on the rate of SDS-stable binding of βA4 by apoE3. Phosphate buffered saline was saturated with O_2 or N_2 prior to incubating apoE3 with $\beta A412_{(1-28)}$ for: Lane 1, 30 min.; Lane 2, 2 hrs; Lane 3, 4 hrs; Lane 4, 6 hrs. βA4 peptide was detected with the anti-βA4 peptide antibody. Upper arrow indicates $\beta A4_{(1-28)}$/apoE complex; lower arrow indicates free βA4.

Figure 10 shows SDS-stable binding of various βA4 peptides to apoE3 and apoE4. ApoE3 and apoE4 were incubated with the indicated concentrations of $\beta A4_{(1-40)}$ (Panel A).; $\beta A4_{(1-28)}$ (Panel B); or $\beta A4_{(12-28)}$ (Panel C) for 5 hrs. The incubation was stopped by the addition of an equal volume of 2X Laemmli buffer (without β-mercaptoethanol), and boiled five minutes. βA4 was detected with the anti-βA4 peptide antibody. Left panels: upper arrows indicate apoE3 dimer/βA4 complex, lower

arrows indicate apoE3 monomer/βA4 complex. Right panels: arrows indicate apoEA/βA4 complex.

Figure 11 shows the pH dependence of SDS-stable βA4 binding by apoE3 and apoE4. ApoE3 and apoE4 were incubated with $\beta A4_{(1-28)}$ in citric acid- Na_2HPO_4 buffer at the indicated pH for 5 hrs. βA4 peptide was detected with the anti-βA4 peptide antibody.

Figure 12 shows the SDS-stable binding of βA4 by truncated apoE3. $\beta A4_{(1-28)}$ was incubated with truncated apoE3 (1 μg) for five hrs. and the incubation ended by boiling in Laemmli buffer (without β-mercaptoethanol) five minutes. Lane 1, $apoE3_{(1-299)}$ (full length); Lane 2, $apoE3_{(1-191)}$; Lane 3, $apoE3_{(1-266)}$; Lane 5, $apoE3_{(1-272)}$. βA4 peptide was detected with the anti-βA4 peptide antibody.

Detailed Description of the Invention

Amino acid sequences disclosed herein are presented in the amino to carboxy direction, from left to right. The amino and carboxy groups are not presented in the sequence.

As noted above, a first aspect of the instant invention is a construct comprising a β-amyloid protein or fragment thereof immobilized on a solid support. Where a fragment is immobilized on the solid support, the fragment is preferably one which binds to amyloid precursor protein binding. Examples of such fragments are the fragments given herein as SEQ ID NO:1 and SEQ ID NO:2, though other fragments, both longer and shorter, may also be employed. The β-amyloid protein or fragment thereof may be immobilized on the support by any suitable means, either directly or indirectly. Examples include affinity binding and covalent binding, with covalent binding currently preferred. Any suitable solid support may be employed, including, but not limited to, nitrocellulose, silica, and polypropylene. The solid support may be in any suitable configuaration, such as particles, beads, wells, or films.

In use, the construct of the present invention may be employed to detect compounds which bind to β-amyloid protein. Generally this involves contacting an aqueous solution containing a compound suspected of binding to the β-amyloid protein to the construct, and then detecting the presence or absence of target compound bound to said construct. Any suitable aqueous solution may be employed, such as a biological fluid (blood serum, cerebrospinal fluid, etc.) or an aqueous solution prepared to contain a specific test compound such as a preselected natural or artificial peptide or analog thereof to be tested for the ability to bind β-amyloid protein. Detecting of the binding event may also be carried out by any suitable means, such as staining, affinity binding, competitive binding assay, etc.

The present invention also provides a method of detecting compounds which inhibit the binding of amyloid precursor protein to β-amyloid protein which involves providing an aqueous solution containing a binding pair [the binding pair comprising *(i)* the amyloid precursor protein or a fragment thereof which binds to the β-amyloid protein, and *(ii)* the β-amyloid protein or a fragment thereof which binds to the amyloid precursor protein], adding a test compound to the aqueous solution; and then

detecting whether or not the test compound inhibits binding between the members of said binding pair. This method is useful as an *in vitro* assay for detecting compounds which may be useful for inhibiting the noted binding event, which is in turn useful in detecting compounds useful for either diagnosing or treating Alzheimer's disease. As above, the detecting step may be carried out by any suitable means, as noted above, and as an aid to detection one member of the binding pair may be immobilized on a solid support. For example, the β-amyloid 1-28 protein fragment having the sequence given herein as SEQ ID NO:1 or the β-amyloid protein fragment comprises the β-amyloid protein 12-28 fragment having the

sequence given herein as SEQ ID NO:2 may be used in *in vitro* assays as described, with the fragments optionally bound to a solid support. The solid support thus may, for example, be a construct as discussed above, though other embodiments (e.g., in which APP or a fragment thereof is immobilized on the solid support) are also suitable.

The present invention also provides a method of inhibiting the binding of amyloid precursor protein to β-amyloid protein which comprises contacting to one member of the amyloid precursor protein-β-amyloid protein binding pair a fragment of the other member of said binding pair (also referred to herein as an "active agent"), as noted above. In a preferred embodiment, the active agent is the β-amyloid 1-28 protein fragment having the sequence given herein as SEQ ID NO:1 or a fragment thereof which binds to amyloid precursor protein, and in a particularly preferred embodiment, the active agent is the β-amyloid protein 12-28 fragment having the sequence given herein as SEQ ID NO:2 or a fragment thereof which binds to amyloid precursor protein. The method may be carried out *in vitro*, as in a diagnostic or screening assay as discussed above, or may be carried out *in vivo* in a human or animal subject to inhibit plaque formation in disorders such as Alzheimer's disease.

As noted above, the present invention also provides a method of detecting compounds which inhibit the binding of β-amyloid protein to apolipoprotein, including apolipoprotein E3 and apolipoprotein E4. The method comprises, first, providing an aqueous solution containing a binding pair, the binding pair comprising *(i)* a first compound selected from the group consisting of Apolipoprotein (e.g., ApoE3, ApoE4) and fragments thereof which bind to the β-amyloid protein, and *(ii)* the β-amyloid protein or a fragment thereof which binds to Apolipoprotein (e.g., ApoE3, ApoE4), then, adding a test compound to said aqueous solution, and then, detecting whether or not the test compound inhibits binding between

the members of the binding pair. This method is useful for the same purposes as described above, and may also be carried out in the same manner as described above.

The present invention also provides a method of inhibiting the binding of apolipoprotein (e.g., ApoE3, ApoE4) to β-amyloid protein. The method comprises contacting to one member of the apolipoprotein-β-amyloid protein binding pair a fragment of the other member of the binding pair (also referred to herein as an "active agent"), as also described above. The method is useful and may be carried out in the same manner as described above (e.g., *in vitro* as in a diagnostic or screening assay, or *in vivo* in a human or animal subject to inhibit plaque formation in disorders such as Alzheimer's disease).

Fragments employed in carrying out the present invention are peptides derived from APP, βA, or Apolipoprotein (e.g., ApoE3, ApoE4) which have N-terminal, C-terminal, or both N-terminal and C-terminal amino acid residues deleted, but retain the biological activity of the parent protein as described herein. Such active fragments may be prepared by enzymatic digestion of βA, APP, or ApoE, by direct synthesis, or by genetic engineering procedures. Fragments employed herein include analogs of βA, APP, ApoE, and the natural sequence active fragments thereof. An "analog" is a chemical compound similar in structure to another which has either a similar or opposite physiological action. Such analogs may be prepared by altering or deleting amino acids. One or more amino acids of a synthetic peptide sequence may be replaced by one or more other amino acids which does not affect the activity of that sequence. Such changes can be guided by known similarities between amino acids in physical features such as charge density, hydrophobicity/hydrophilicity, size and configuration. For example, Thr may be replaced by Ser and vice versa, Asp may be replaced by Glu and vice versa, and Leu may be

replaced by Ile and vice versa. Further, those skilled in this art will appreciate that minor changes may be made to the naturally occuring amino acids to produce derivatives thereof which retain activity.

Fragments of the proteins and peptides described herein include the analogs thereof, as noted above. As used herein, analogs are those compounds which, while not having amino acid sequences identical to those of the peptides described above, have a similar three-dimensional structure. In molecules which interact with a specific binding partner, the interaction between the two binding partners must take place at the surface-accessible sites in a stable three-dimensional molecule. By arranging the critical binding site residues in an appropriate conformation, it is possible to synthesize compounds which mimic the essential surface features of the binding partner. A molecule which has a surface region with essentially the same molecular topology to the binding surface of one binding partner will be able to mimic the interaction of that binding partner with its specific binding partner. Methods for determining peptide three-dimensional structure and analogs thereto are known, and are sometimes referred to as "rational drug design techniques". *See, e.g.*, U.S. Patent No. 4,833,092 to Geysen; U.S. Patent No. 4,859,765 to Nestor; U.S. Patent No. 4,853,871 to Pantoliano; U.S. Patent No. 4,863,857 to Blalock; (the disclosures of which are incorporated by reference herein in their entirety); *see also* Waldrop, *Science* **247**, 28029 (1990); Rossman, *Nature* **333**, 392-393 (1988); Weis et al., *Nature* **333**, 426-431 (1988). For example, techniques for constructing and screening libraries of peptide sequences to identify peptides that specifically bind to a given protein are described in Scott and Smith, *Science* **249**, 386-390 (1990) and in Devlin et al., *Science* **249**, 404-406 (1990).

The amount of active agent administered to a subject will vary depending upon the age, weight,

condition of the subject, and the particular disorder or disorders being treated, but is generally from .1 nanograms to 10 micrograms, and is typically an amount ranging from 1 nanogram to 1 microgram.

Pharmaceutical compositions containing the active agents of the present invention may be prepared in either solid or liquid form. To prepare the pharmaceutical compositions of this invention, one or more compounds of salt thereof of the invention as the active ingredient, is intimately admixed with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques, which carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g. oral, inhalation or parenteral. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed. Thus, for liquid oral preparation, such as for example, suspensions, elixirs and solutions, suitable carriers and additives include water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like; for solid oral preparations such as, for example, powders, capsules and tablets, suitable carriers and additives include starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like. Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar coated or enteric coated by standard techniques. For parenterals, the carrier will usually comprise sterile water, though other ingredients, for example, for purposes such as aiding solubility or for preservatives, may be included. Injectable suspensions may also be prepared, in which case appropriate liquid carriers, suspending agents and the like may be employed.

Active agents of the present invention may be administered per se or in the form of a pharmaceutically

acceptable salt. Such pharmaceutically acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, salicylic, p-toluenesulfonic, tartaric, citric, methanesulphonic, formic, malonic, succinic, naphthalene-2-sulphonic and benzenesulphonic. Also, pharmaceutically acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of a carboxylic acid group.

The present invention is explained in detail in the Examples set forth below. These Examples are for illustrative purposes only, and are not to be taken as limiting of the invention.

EXAMPLE 1

Immobilization of βA Fragments on Solid Support

$βA_{(1-28)}$ peptide (Bachem) (SEQ ID NO:1) or ethanolamine was covalently immobilized to Immobilon AV affinity membrane (Millipore). The membrane is a chemically activated hydrophilic microporous membrane which covalently immobilizes peptides and proteins through amino and thiol groups. βA peptide was dissolved in distilled water at one mg peptide/100 μl. Ten microliters (containing one hundred micrograms peptide) were applied to a 13 mm diameter Immobilon disc, and incubated to dryness overnight at room temperature. Peptide was in large excess to the number of functional binding groups on the membrane. Control membranes, containing no peptide, were prepared by incubating the membrane in 2.0 M ethanolamine in 1.0 M $NaHCO_3$, pH 9.5, to block the reactive groups on the Immobilon AV membrane. Membranes were stored at -20° C in a dessicator. Prior to use the membranes were washed with phosphate buffered saline.

EXAMPLE 2

Binding of CSF Proteins to Immobilized βA(1-28)

The binding of proteins in human cerebrospinal fluid to $\beta A_{(1-28)}$ peptide or to ethanolamine immobilized to Immobilon AV membranes, prepared as described in Example 1 above, is shown in **Figure 1.** Immobilon AV membranes previously bound with $\beta A_{(1-28)}$ peptide (βA) or with ethanolamine (C) were incubated with 100 μl cerebrospinal fluid with 50 μl phosphate-buffered saline pH 7.3 for thirty minutes at room temperature. After incubation the membranes were placed in a Millipore filter holder (Swinnex) and washed with 3.0 ml phosphate-buffered saline (Bracket 1), and then washed with 700 μl 10% sodium dodecyl sulfate (SDS)(Bracket 2), 700 μl 4 M urea (Bracket 3), or 700 μl 6 M guanidine hydrochloride (Bracket 4). The membranes were removed from the filter holder, cut in half and placed in 150 μl of Laemmli buffer (2% sodium dodecylsulfate, 5% beta-mercaptoethanol, pH 6.8) and boiled five minutes to solubilize retained proteins. Forty-five μl of Laemmli buffer with solubilized protein were loaded in each of ten lanes of a Bio-Rad Minigel apparatus, with a stacking gel of 4%, and a separating gel of 7.5% polyacrylamide with 2% SDS. In (A) the gel was silver stained. In (B) electrophoresed proteins from an identically prepared gel were transferred to Immobilon P, using standard Western transfer techniques. After transfer the membrane was incubated in Blotto (5% dried milk in Tris buffered saline, pH 7.6, with 0.5% Tween-20 (Pierce) at room temperature for one hour. The membrane was next incubated in the Anti-Alzheimer amyloid precursor antibody (mouse monoclonal : clone 22C11) from Boehringer Mannheim (at 1:200 dilution) in Blotto overnight at 4° C, then washed five times in Blotto. The membrane was exposed to secondary antibody conjugated with horseradish peroxidase for one hour at room temperature, then washed seven times in Blotto. Horseradish peroxidase was then

visualized with Enhanced Chemiluminesce Detection kit (Amersham), and exposed to Hyperfilm ECL (Amersham).

These data indicated that many proteins in human cerebrospinal fluid were found to bind to synthetic ßA peptide amino acids 1-28 ($ßA_{(1-28)}$) immobilized to Immobilon AV membrane, and do not bind to control membrane with covalently-bound ethanolamine (**Figure 1A**; Bracket 1). Of these cerebrospinal fluid proteins, many bind ßA peptide with high affinity, since they are only partially eluted with 10% sodium dodecyl sulfate (SDS) (**Figure 1A**; Bracket 2), 4 M urea (Bracket 3), or 6 M guanidine hydrochloride (Bracket 4).

Since cerebrospinal fluid contains secreted APP, we wanted to determine whether APP was one of the proteins binding ßA peptide in vitro. Although a number of proteins bound to both ßA peptide and control membranes (**Figure 1A**), APP was found only among the proteins bound to the ßA peptide membranes (**Figure 1B**). Immunodetected APP have the molecular weights of the secreted forms of APP previously described in cerebrospinal fluid. M. Palmert, *Proc. Natl. Acad. Sci. USA* **86**, 6338-6342 (1989). APP retained by immobilized ßA peptide (1-28) is only partially eluted by 10% SDS (**Figure 1B**; Bracket 2) and 6 M guanidine hydrochloride (Bracket 4), and is not eluted by 4 M urea (Bracket 3).

EXAMPLE 3

Binding of CSF Proteins to Immobilized *ßA*(12-28)

The binding of proteins in human cerebrospinal fluid proteins to $ßA_{(12-28)}$ peptide (SEQ ID NO:2), or to the control 12-28 Hydropathic Mimic Peptide (H.M.) (SEQ ID NO:3) immobilized to Immobilon AV membranes, is shown in **Figure 2**. Membranes were prepared in essentially the same manner as given in Example 1. In overview, 100 μl of human cerebrospinal fluid in 50 μl phosphate buffered saline was incubated thirty minutes with Immobilon AV membranes previously prepared with immobilized $ßA_{(12-28)}$

peptide or with immobilized 12-28 Hydropathic Mimic Peptide (H.M.). Membranes were then washed and prepared identically to those shown in Figure 1: Bracket 1, Phosphate-buffered saline; Bracket 2, 10% SDS; Bracket 3, 4M urea; Bracket 4, 6M guanidine hydrochloride. Proteins were electrophoresed and then visualized by silver staining (Figure 2A) or immunodetection with Anti-Alzheimer precursor protein antibody following Western transfer to Immobilon P (Figure 2B).

The data collected showed that the shorter βA peptide (amino acids 12-28) given in SEQ ID NO:2 also bound APP. Many cerebrospinal fluid proteins bound to both immobilized peptides after washing the membranes with phosphate buffered saline (**Figure 2A**; Bracket 1). Several proteins remained bound to the $\beta A_{(12-28)}$ peptide, but were eluted from the control H.M. peptide by 10% SDS (Bracket 2), 4 M urea (Bracket 3), or 6 M guanidine hydrochloride (Bracket 4). Conversely, the immobilized control peptide (H.M.) retained several proteins which were eluted from immobilized $\beta A_{(12-28)}$ peptide following wash with 6 M guanidine hydrochloride (Bracket 4).

Binding of APP to immobilized $\beta A_{(12-28)}$ peptide or to immobilized H.M. control peptide is shown in **Figure 2B**. APP was retained by both the $\beta A_{(12-28)}$ peptide and the control peptide following wash with phosphate buffered saline (Bracket 1). 10% SDS eluted little APP from $\beta A_{(12-28)}$ peptide, but eluted most of the APP from the control peptide (Bracket 2). Similarly, guanidine hydrochloride eluted little APP from $\beta A_{(12-28)}$ peptide, but eluted virtually all detectable APP from the H.M. control peptide (Bracket 4).

EXAMPLE 4

Preparation of Human APP Isoforms and Deletion Mutants

Various APP isoforms and deletion mutants produced in baculovirus-transformed insect cells are

schematically illustrated in **Figure 3**. These cells synthesize and release into the culture media human recombinant APP after proteolytically processing the precursor protein at the same peptide bond hydrolyzed by mammalian cells. *See* R. Bhasin et al., *Proc. Natl. Acad. Sci. USA.* **88**, 10307-10311 (1991); D. Lowery et al., *J. Biol. Chem.* **266**, 19842-19850, (1991).

APP-695, APP-751, and APP-770 were produced by known techniques. *See* R. Bhasin et al., *supra*. RIS were created by digestion of full length clones of APP with EcoR1, filling in the 5' - overhangs with Klenow, and self ligation, in accordance with known techniques. *See generally* T. Maniatis et al., in *Molecular Cloning: A Laboratory Manual*, (Cold Spring Harbor Laboratory, N.Y. 1989). This frameshift results in a stop codon seven amino acids after the start of the ßA domain. The last three amino acids before the stop condon were altered in the process. $\mu\Delta$ were created by digestion of full length clones of APP with XhoI and BglII followed by filling in of the overhangs by Klenow. *See generally* T. Maniatis et al., *supra*. The 5 Kb top fragment was gel purified and self ligated, creating a deletion of 295 amino acids between the Kunitz protease inhibitor domain and the ßA domain. K mutants were created by digestion of the corresponding APP $\mu\Delta$ plasmids with XhoI followed by filling in of the 5' - overhangs with Klenow and self ligation. The resultant plasmids contained a stop condon after 308 amino acids in APP695-K, and 383 amino acids in APP770-K. Three amino acids before the stop codon were altered in the process. KXΔ was created by digestion of full length APP with KpnI and XcmI, followed by digestion of the 3' - overhangs with T4 DNA polymerase. The ~5 Kb bands were gel purified and self ligated. The resultant plasmids had 263 amino acids deleted after amino acid 19 of APP, thereby deleting the cysteine rich and negatively charged domains of APP.

All deleted APP clones were subsequently transferred to the baculovirus expression plasmid pJVP10 in accordance with known techniques. *See* R. Bhasin et al., *Proc. Natl. Acad. Sci. USA.* **88**, 10307-10311 (1991). Baculovirus expression plasmids containing the deleted APPs were co-transfected with either AcNPV DNA or linearized RP6 baculovirus DNA (for APP μ_Δ and APP-K clones) as previously described. *See* R. Bhasin et al., *supra;* P. Kitts et al., *Nucl. Acid Res.* **18**, 5667-5672, (1990).

EXAMPLE 5

Binding of Human APP Isoforms to Immobilized βA(1-28)

The binding of recombinant-expressed human APP isoforms to $\beta A_{(1-28)}$ peptide or to ethanolamine immobilized to Immobilon AV is illustrated in **Figure 4.**

In **Figure 4A**, conditioned media (20 μl) from baculovirus-infected insect cells transfected with the genes for APP-695, APP-751 or APP-770 (in 130 μl phosphate-buffered saline) prepared as described in Example 4 above were incubated with Immobilon AV membranes previously prepared with $\beta A_{(1-28)}$ peptide (βA), or with ethanolamine (C), then washed with 3.0 ml phosphate buffered saline and 700 μl 10% SDS. The membranes were then boiled five minutes in Laemmli buffer and the eluted proteins electrophoresed. Lanes marked STD are 4 μl of conditioned media in Laemmli buffer loaded directly on the gel. Following electrophoresis, proteins were transferred to Immobilon P by Western technique, and the APP isoforms detected by the Anti-Alzheimer precursor protein antibody, and visualized as described previously.

In **Figure 4B**, binding of APP deletion mutants prepared as describedin in Example 5 above to $\beta A_{(1-28)}$ peptide, or to ethanolamine (C) immobilized on Immobilon AV was examined. 20 μl of conditioned media from baculovirus-infected cells expressing the deletion mutant APP indicated, in phosphate buffered saline (total volume

160 μl), were incubated with immobilized $\beta A_{(1-28)}$ peptide (βA) or ethanolamine (C). The membranes were washed, the retained proteins eluted in Laemmli buffer, electrophoresed and detected with the Anti-Alzheimer precursor protein antibody, as described previously.

As shown in **Figure 4A**, APP-695, APP-751, and APP-770 was retained by membranes with immobilized $\beta A_{(1-28)}$ peptide, but was eluted from control membranes following 10% SDS. Since the APP-695 isoform bound to the $\beta A_{(1-28)}$ peptide, the Kunitz domain and a unique 19 amino acid region in APP-770 do not appear to be required for APP binding.

To further identify the region of APP involved in the binding to βA peptide, deletion mutants of APP were tested. Mutants of APP containing only the amino terminus of APP (695-K and 770-K) were examined for βA binding, to determine whether the amino terminus of APP is capable of binding βA peptide. As shown in **Figure 4B**, these amino-terminus fragments of APP bound to the immobilized βA peptide, but not to the control membrane. The RIS mutant terminates after the seventh amino acid of the amyloid domain, with substitution of three unrelated amino acids, and also bound to immobilized $\beta A_{(1-28)}$ peptide. The μ_{Δ} deletion mutant lacks the peptide region between the Kunitz domain and βA amyloid, and similarly bound to $\beta A_{(1-28)}$ peptide. The KX$_{\Delta}$ deletion mutants lack the amino terminus of APP between the signal sequence and the Kunitz domain.

EXAMPLE 6

<u>Binding of APP Deletion Mutants to Immobilized $\beta A(1-28)$</u>

The binding of 770 APP and deletion APP isoforms 770 KX$_{\Delta}$ and 751 KX$_{\Delta}$ to $\beta A_{(1-28)}$ peptide (βA) and to ethanolamine-control (C) immobilized Immobilon AV membranes is illustrated in **Figure 5**. Conditioned media from baculovirus-infected insect cells expressing 770-APP (20μl) or the deletion APP mutations 770 KX$_{\Delta}$ (20μl) and 751 KX$_{\Delta}$ (4μl) were incubated in phosphate-buffered saline

(total volume 150 μl) with Immobilon AV membranes previously bound with $\beta A_{(1-28)}$ or ethanolamine (C). The membranes were washed with phosphate buffered saline and 10% SDS, as described previously. Gel lanes marked STD were loaded with conditioned media in Laemmli (4 μl of 770-APP and 770-KX$_\Delta$ and 0.8 μl of 751-KX$_\Delta$). After boiling the membranes in Laemmli buffer, the eluted proteins were electrophoresed, transferred by Western technique and visualized by the Alpha-5-anti-Alzheimer amyloid precursor protein antibody, a rabbit polyclonal antibody directed against amino acids 444-595 of APP-695, produced and graciously supplied by Dr.Ivan Lieberburg. Increasing the exposure time of the Hyperfilm with the immunoblot by five times, as shown in **Figure 5B**, demonstrates small amounts of the KX$_\Delta$ mutants bound to βA peptide, not detected on the ethanolamine-control membranes.

Binding of KX$_\Delta$ to $\beta A_{(1-28)}$ peptide was barely detectable (**Figure 5A**). These observations suggest that the binding of APP to $\beta A_{(1-28)}$ peptide occurs within the amino terminus part of the protein, between the signal sequence and the Kunitz domain. Increasing exposure time of the immunoblot, shown in **Figure 5B**, demonstrated that small amounts of APP-751 KX$_\Delta$ and APP-770 KX$_\Delta$ bound to $\beta A_{(1-28)}$ peptide and not to the control membrane. This small amount of binding of the KX$_\Delta$ isoforms to $\beta A_{(1-28)}$ peptide may be due to a weaker binding domain elsewhere in the protein, or may be due to possible microheterogeneity of the recombinant proteins.

EXAMPLE 7

Effect of Reducing Conditions on Binding of APP Isoform 695-K to Immobilized βA(1-28)

The effect of reducing disulfide bonds, and of pH, on the binding of 695-K to $\beta A_{(1-28)}$ peptide is shown in **Figure 6.**

In **Figure 6A**, Disulfide bonds of the 695-K deletion mutant were reduced by boiling 100 μl of

conditioned media in 10 μl of 300 mM dithiothreitol for five minutes. After cooling, the conditioned media was incubated in the dark with 10 μl iodoacetic acid (20 mg in 100 μl distilled water) at 37° C for thirty minutes. 30 μl of this material (Reduced) or 20 μl untreated material (Control) in 140 μl phosphate buffered saline were incubated with immobilized $\beta A_{(1-28)}$ (βA), or ethanolamine (C) and the membranes were washed with phosphate buffered saline and 10% SDS. The retained proteins were then eluted in Laemmli buffer as described previously. The lanes marked STD (standards) were loaded directly with 6 μl of treated or 4 μl of control conditioned media. After polyacrylamide gel electrophoresis, the proteins were transferred to Immobilon P, and detected with the Anti-Alzheimer precursor protein antibody. (BOTTOM) To determine the effects of pH on binding, 20 μl of conditioned media containing the 695-K deletion mutant in 130 μl of 0.1 M citric acid , 0.2 M Na_2HPO_4 at the indicated pH, were incubated with immobilized $\beta A_{(1-28)}$ peptide thirty minutes. The membranes were washed and eluted with 10 % SDS, and the 695-K APP was detected on the immunoblot following electrophoresis and transfer, as described above.

The 695-K mutant APP, which binds βA peptide, is the amino terminus domain of APP containing twelve cysteines and the negatively-charged region rich in glutamic (28 residues) and aspartic (17 residues) acids. To determine whether the tertiary structure of this peptide established by disulfide bridges was necessary for binding βA peptide, the disulfide bonds were reduced. As shown in **Figure 6A**, reduction of disulfide bonds did not prevent binding of the 695-K mutant to βA peptide, suggesting that this binding does not require large domains of the protein with native tertiary structure. To determine whether charge interactions between the 695-K mutant and βA peptide were important in binding, binding was assayed over the pH range 2.5 to 7.6. The calculated

pI of the 695-K mutant is 3.98. As shown in **Figure 6B**, binding between 695-K mutant and $\beta A_{(1-28)}$ was dependent on pH, with increased binding observed at pH values lower than 7.0.

EXAMPLE 8

Isolation of apoE3 and apoE4

The apoE3 and apoE4 isoforms were isolated from the plasma of fasting subjects with the E3/3 and E4/4 homozygous phenotypes, using techniques previously described in S. Rall, *Methods Enzymol.* **128**, 273 (1986). Proteolytic fragments of apoE as described in T. Innerarity, *J. Biol. Chem.* **258**, 12341 (1983), and recombinant-expressed truncated apoE were produced according to methods known to those skilled in the art. Synthetic βA4 peptides were obtained from Bachem. One mg βA4 peptide was dissolved in 60μl distilled water, then diluted with phosphate buffered saline, pH 7.30 as needed.

EXAMPLE 9

Formation of apoE-βA4 Complex

One μg of purified apoE was incubated five hours at 37°C with βA4 peptide in phosphate buffered saline, pH 7.30, in a total volume of 20μl. The incubation was terminated by the addition of two 20μl aliquots of Laemmle buffer (4% SDS, with no β-mercaptoethanol), and boiled five minutes. Samples were stored at -80°C. Proteins were electrophoresed on either a 7.5 or 12% polyacrylamide gel with 2% sodium dodecyl sulfate and transferred to Immobilon P, as previously described in W.J. Strittmatter, *Proc. Natl. Acad. Sci. USA*, **90**:1977 (1993). The Immobilon membrane was washed and incubated in primary antibody overnight, as described in W.J. Strittmatter, *Proc. Natl. Acad. Sci. USA*, **90**:1977 (1993). Rabbit-anti human apoE antibody was used at 1:5000. Rabbit-anti βA4 amyloid peptide antibody was used at 1:80. The Immobilon membrane was incubated with horseradish peroxidase-conjugated

secondary antibody, and chemiluminescence was visualized by exposure to Hyperfiled obtained from Amersham. Quantitative scanning densitometry was on a Hoeffer gel scanner, and was analyzed with the included GS370 software.

Incubation of purified, delipidated apoE4 or apoE3 with synthetic βA4 peptide ($\beta A4_{(1-28)}$) resulted in the formation of an apoE-βA4 peptide complex that was recognized by both an apoE antibody and by a βA4 peptide antibody. This complex was maintained even after boiling in 2% SDS for five minutes. See **Figure 7**. In contrast, boiling apoE prior to incubation with βA4 peptide prevented binding (data not shown). The apoE3/βA4 peptide complex was first detectable after two hours incubation, and increased over the next twenty-four hours. In contrast the apoE4/βA4 complex was easily detected after five minutes incubation. βA4 peptide bound to both the monomer of apoE3, and to the disulfide-linked homodimer of apoE3. See **Figures 8** and **10**. After incubation for twelve hours, an additional, higher molecular weight apoE/βA4 complex was observed. Only a small percentage (less than 10%) of the total amount of apoE in the incubation bound βA4 peptide after twenty four hours, despite a large molar excess of βA4 peptide (βA4 peptide at 2.5×10^{-4} molar, apoE at 1.8×10^{-6} molar).

EXAMPLE 10

Effects of Reducing Agents β-Mercaptoethanol or Dithiothreitol on SDS-Stable Binding by βA4 by apoE

One μg apoE3 or apoE4 were incubated with $\beta A4_{(1-28)}$ (2.5×10^{-4} M) for five hours at 37°C. To one sample, 9.2% (V/V) β-mercaptoethanol was added during incubation. To a second sample, 30 mM dithiothreitol was added during the incubation. The incubation was stopped by the addition of two aliquots of an equal volume of Laemmle buffer (without β-mercaptoethanol), and boiled five minutes. β-mercaptoethanol was added to a third sample after the

incubation. Dithiothreitol was added to a fourth sample after incubation. Proteins were electrophoresed on a 10% polyacrylamide gel, transferred to Immobilon P membrane, and the βA4 peptide/apoE complexes were detected by anti-βA4 peptide antibody.

Addition of the reducing agents dithiothreitol or β-mercaptoenthanol, either before or after incubation of apoE and βAE peptide, prevented SDS-stable binding, see **Figure 8**, suggesting that oxidation may be required. No differences were detected in the amount, or relative molecular weight, of immunoreactive apoE during these incubations (not shown).

EXAMPLE 11

Effect of O_2 and N_2 on the Rate of SDS-Stable Binding of βA4 by apoE3

Phosphate buffered saline was saturated with O_2 or N_2 prior to the incubation of apoE3 with $\beta A4_{(1-28)}$. βA4 peptide was detected with the anti-βA4 peptide antibody.

As shown in **Figure 9**, oxygen increased and nitrogen decreased the rate of SDS-stable binding. Incubation of apoE3 or apoE4 alone in oxygenated buffer increased the amount of βA4 peptide bound subsequently (data not shown). Prolonged incubation of apoE3 or apoE4 alone at 37°C resulted in the gradual loss of the ability of apoE to bind βA4 peptide. The amount of immunoreactive apoE was unchanged during these incubations. These results demonstrate that both apoE3 and apoE4 bind βA4 peptide, forming a complex which resists dissociation by boiling in SDS. Binding of βA4 by apoE appears to require the oxidation of apoE, and can be prevented or reversed by reduction with dithiothreitol or β-mercaptoethanol.

EXAMPLE 12

SDS-stable binding of various βA4 peptides to apoE3 and apoE4

ApoE3 and apoE4 were incubated with various concentrations of $\beta A4_{(1-40)}$, $\beta A4_{(1-28)}$, or $\beta A4_{(12-28)}$ for five hours. The incubation was stopped by the addition of two aliquots of an equal volume of Laemmli buffer (without β-mercaptoethanol) and boiled five minutes. βA4 was detected with the anti-βA4 peptide antibody.

The SDS-stable binding of βA4 peptides by apoE4 and by apoE3 was dose dependent. As shown in **Figure 10**, apoE3 and apoE4 bound $\beta A4_{(1-40)}$, $\beta A4_{(1-28)}$, and $\beta A4_{(12-28)}$ and was maximal at 10^{-4} molar peptide in all three cases and half-maximal binding was approximately 10^{-5} molar.

EXAMPLE 13

pH dependence of SDS-stable βA4 binding by apoE3 and apoE4

ApoE3 and apoE4 were incubated with $\beta A4_{(1-28)}$ in citric acid-Na_2HPO_4 buffer for five hours. βA4 peptide was detected with the anti-βA4 peptide antibody. The domain of apoE which binds βA4 peptide was determined by examining various apoE fragments. Proteolysis of apoE by thrombin produces a 22-kDa fragment containing amino acid residues 1 through 191 as described in T.L. Innerarity, et al., *J. Biol. Chem.* **258**:12341 (1983). $\beta A4_{(1-28)}$ was incubated with 1μg truncated apoE3 for five hours and the incubation ended by boiling in Laemmli buffer (without β-mercaptoethanol) five minutes. βA4 peptide was detected with the anti-βA4 peptide antibody.

βA4 peptide did not bind to the 22-kDa apoE3 fragment. See **Figure 12**. Binding of βA4 peptide to $apoE3_{(1-244)}$ was very low or minimal. In contrast, $apoE3_{(1-266)}$ did form SDS-stable βA4 peptide complex, which was further increased with $apoE_{(1-272)}$. See **Figure 12**. Therefore, βA4 binding by apoE appears to require the domain of apoE between amino acids 244 to 272 within the region

previously demonstrated to mediate binding to lipoprotein particles as suggested in J.A. Westerlund, et al., *J. Biol. Chem.* **263**:6249 (1988).

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Strittmatter, Warren J.

(ii) TITLE OF INVENTION: Method of Inhibiting Binding of Amyloid Precursor Protein to Beta-Amyloid Protein

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Kenneth D. Sibley; Bell, Seltzer, Park and Gibson
(B) STREET: Post Office Drawer 34009
(C) CITY: Charlotte
(D) STATE: North Carolina
(E) COUNTRY: U.S.A.
(F) ZIP: 28234

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Sibley, Kenneth D.
(B) REGISTRATION NUMBER: 31,665

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 919-881-3140
(B) TELEFAX: 919-881-3175
(C) TELEX: 575102

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys
1 5 10 15

Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
20 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Val His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn
1 5 10 15

Lys

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu Gln Gln His Lys Val Leu Ile Ile Thr Asp Glu Leu Gly Thr Gln
1 5 10 15

Lys

THAT WHICH IS CLAIMED IS:

1. A construct comprising a β-amyloid protein or fragment thereof immobilized on a solid support.

2. A construct according to claim 1, wherein said β-amyloid protein or fragment thereof comprises β-amyloid protein.

3. A construct according to claim 1, wherein said β-amyloid protein or fragment thereof comprises a β-amyloid protein fragment which binds to amyloid precursor protein.

4. A construct according to claim 3, wherein said β-amyloid protein fragment comprises the β-amyloid 1-28 protein fragment having the sequence given herein as SEQ ID NO:1.

5. A construct according to claim 3, wherein said β-amyloid protein fragment comprises the β-amyloid protein 12-28 fragment having the sequence given herein as SEQ ID NO:2.

6. A construct according to claim 1, wherein said β-amyloid protein or fragment thereof is covalently bound to said solid support.

7. A construct according to claim 1, wherein said solid support comprises a membrane.

8. A method of detecting compounds which bind to β-amyloid protein, comprising:

providing a construct comprising a β-amyloid protein or fragment thereof immobilized on a solid support;

contacting an aqueous solution containing a compound suspected of binding to the β-amyloid protein to said construct; and

detecting the presence or absence of target compound bound to said construct.

9. A method according to claim 8, wherein said aqueous solution is cerebrospinal fluid.

10. A method of detecting compounds which inhibit the binding of amyloid precursor protein to β-amyloid protein, comprising:

providing an aqueous solution containing a binding pair, said binding pair comprising *(i)* the amyloid precursor protein or a fragment thereof which binds to the β-amyloid protein, and *(ii)* the β-amyloid protein or a fragment thereof which binds to the amyloid precursor protein;

adding a test compound to said aqueous solution; and then

detecting whether or not said test compound inhibits binding between the members of said binding pair.

11. A method according to claim 10, wherein one member of said binding pair is immobilized on a solid support.

12. A method according to claim 10, wherein said β-amyloid protein or fragment thereof which binds amyloid precursor protein is immobilized on a solid support.

13. A method according to claim 12, wherein said β-amyloid protein or fragment thereof comprises a β-amyloid protein fragment containing the amyloid precursor protein binding domain.

14. A method according to claim 13, wherein said β-amyloid protein fragment comprises the β-amyloid 1-28 protein fragment having the sequence given herein as SEQ ID NO:1.

15. A method according to claim 13, wherein said β-amyloid protein fragment comprises the β-amyloid protein 12-28 fragment having the sequence given herein as SEQ ID NO:2.

16. A method according to claim 13, wherein said test compound is selected from the group consisting of β-amyloid protein fragments and amyloid precursor protein fragments.

17. A method of inhibiting the binding of amyloid precursor protein to β-amyloid protein, comprising:

contacting to one member of the amyloid precursor protein-β-amyloid protein binding pair a fragment of the other member of said binding pair, wherein said fragment binds to said one member, and wherein said fragment is provided in an amount sufficient to inhibit binding of amyloid precursor protein to β-amyloid protein.

18. A method according to claim 17, wherein said one member is β-amyloid protein and said fragment is an amyloid precursor protein fragment.

19. A method according to claim 17, wherein said one member is amyloid precursor protein and said fragment is a β-amyloid protein fragment.

20. A method according to claim 19, wherein said β-amyloid protein fragment comprises the β-amyloid 1-28 protein fragment having the sequence given herein as

SEQ ID NO:1 or a fragment thereof which binds to amyloid precursor protein.

21. A method according to claim 19, wherein said β-amyloid protein fragment comprises the β-amyloid protein 12-28 fragment having the sequence given herein as SEQ ID NO:2 or a fragment thereof which binds to amyloid precursor protein.

22. A method of detecting compounds which inhibit the binding of β-amyloid protein to apolipoprotein, comprising:

providing an aqueous solution containing a binding pair, said binding pair comprising *(i)* a first compound selected from the group consisting of apolipoprotein and fragments thereof which bind to the β-amyloid protein, and *(ii)* the β-amyloid protein or a fragment thereof which binds to apolipoprotein;

adding a test compound to said aqueous solution; and then

detecting whether or not said test compound inhibits binding between the members of said binding pair.

23. A method according to claim 22, wherein one member of said binding pair is immobilized on a solid support.

24. A method according to claim 22, wherein said β-amyloid protein or fragment thereof is immobilized on a solid support.

25. A method according to claim 22, wherein said β-amyloid protein or fragment thereof comprises a β-amyloid protein fragment containing the amyloid precursor protein binding domain.

26. A method according to claim 25, wherein said β-amyloid protein fragment comprises the β-amyloid 1-28 protein fragment having the sequence given herein as SEQ ID NO:1.

27. A method according to claim 25, wherein said β-amyloid protein fragment comprises the β-amyloid 12-28 protein fragment having the sequence given herein as SEQ ID NO:2.

28. A method according to claim 22, wherein said test compound is selected from the group consisting of β-amyloid protein fragments.

29. A method according to claim 22, wherein said apolipoprotein is apolipoprotein E3.

30. A method according to claim 22, wherein said apolipoprotein is apolipoprotein E4.

31. A method of inhibiting the binding of apolipoprotein to β-amyloid protein, comprising:

contacting to one member of the apolipoprotein - β-amyloid protein binding pair a fragment of the other member of said binding pair, wherein said fragment binds to said one member, and wherein said fragment is provided in an amount sufficient to inhibit binding of apolipoprotein to β-amyloid protein.

32. A method according to claim 31, wherein said one member is β-amyloid protein and said fragment is an apolipoprotein fragment.

33. A method according to claim 31, wherein said one member is apolipoprotein and said fragment is a β-amyloid protein fragment.

34. A method according to claim 33, wherein said β-amyloid protein fragment comprises the β-amyloid 1-28 protein fragment having the sequence given herein as SEQ ID NO:1.

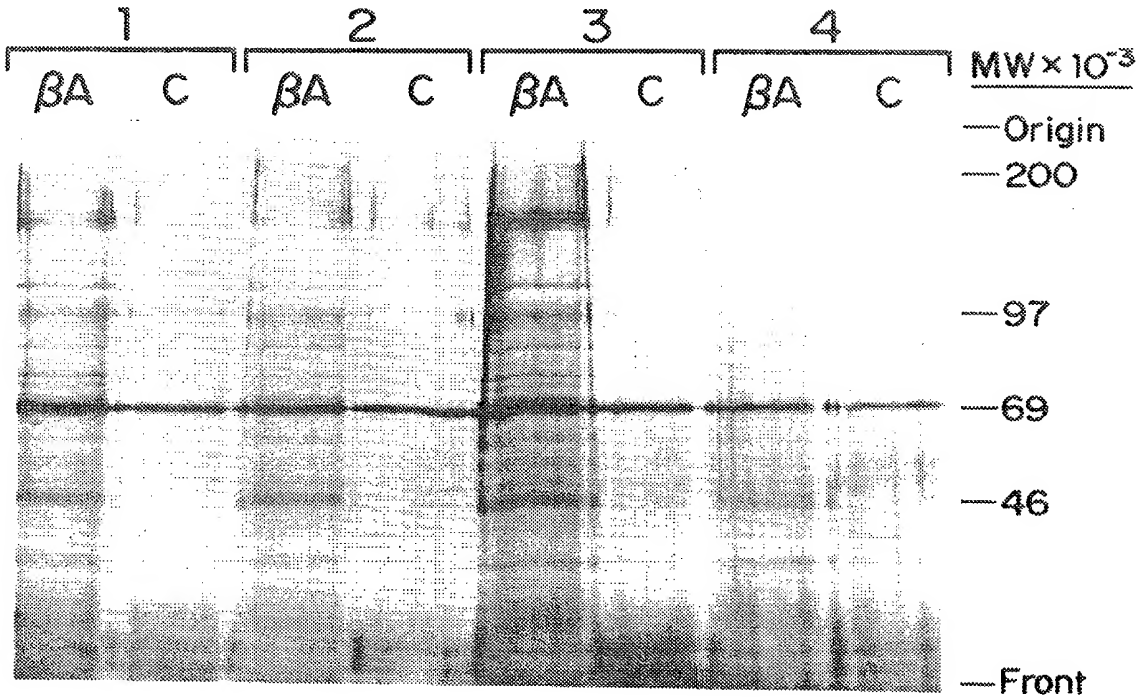
35. A method according to claim 33, wherein said β-amyloid protein fragment comprises the β-amyloid 12-28 protein fragment having the sequence given herein as SEQ ID NO:2.

36. A method according to claim 31, wherein said apolipoprotein is apolipoprotein E3.

37. A method according to claim 31, wherein said apolipoprotein is apolipoprotein E4.

1/12

FIG. 1A



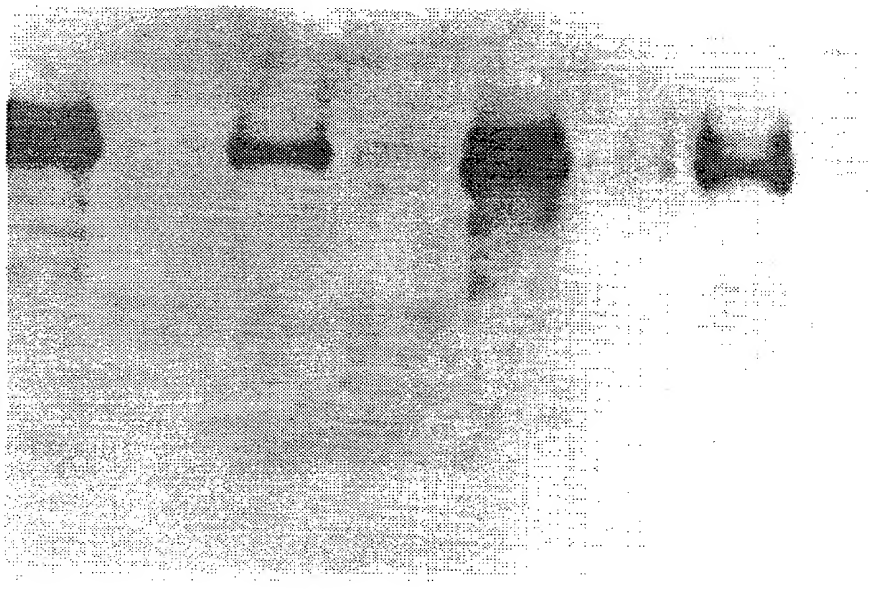
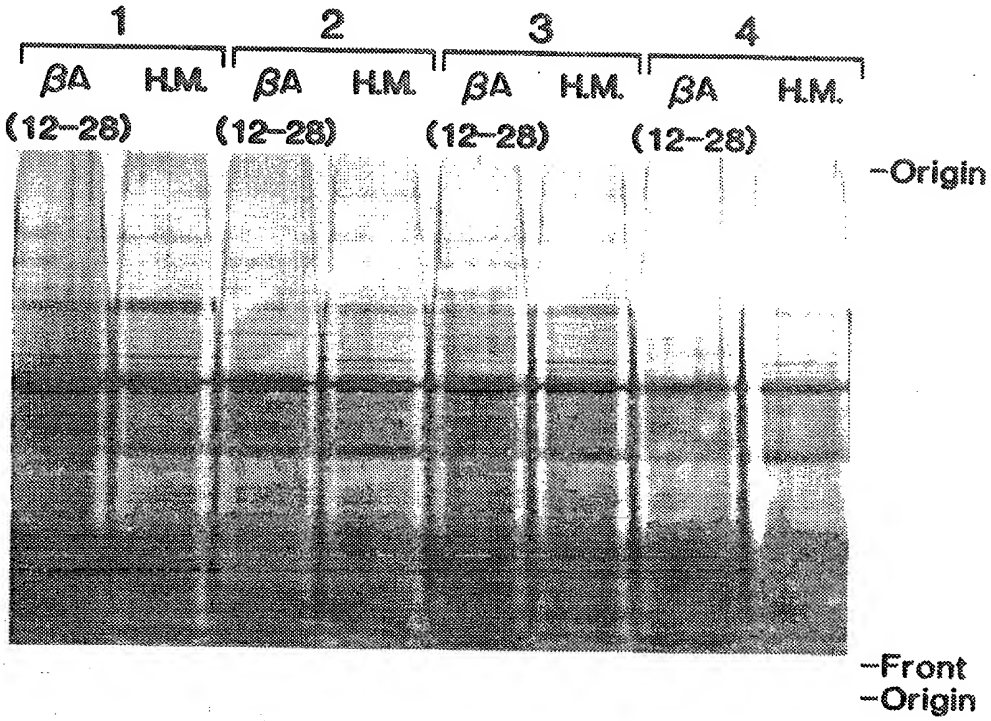


FIG. 1B

FIG. 2A


1
2
3
4
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H.M.
βA
H.M.
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H.M.
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(12–28)
(12–28)
(12–28)
–Origin
–Front
–Origin

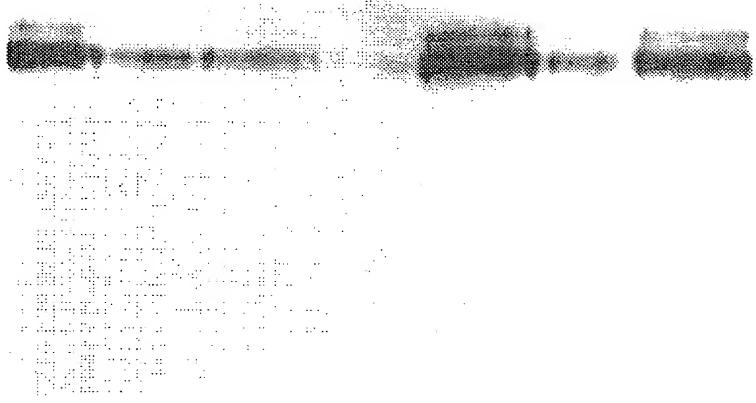
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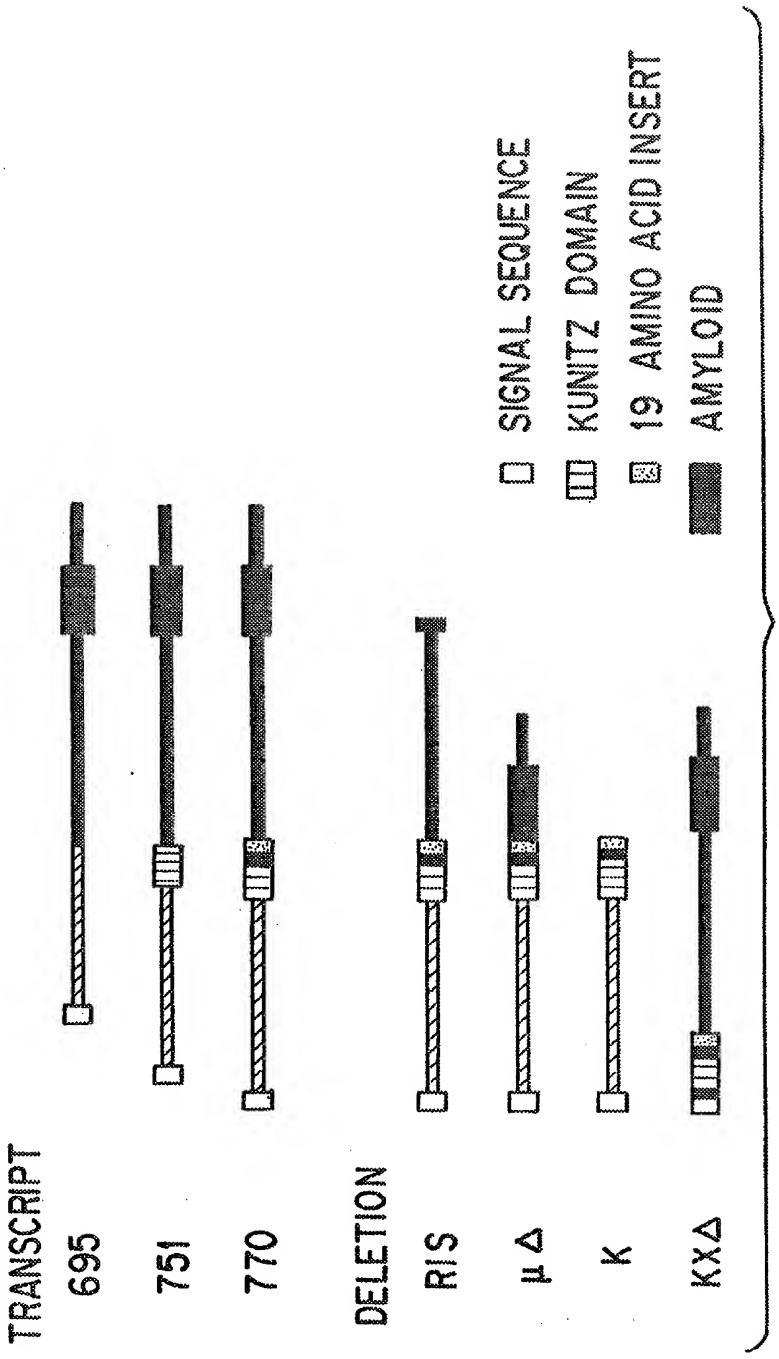
FIG. 2B


TRANSCRIPT
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751
770
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RIS
μ Δ
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KXΔ
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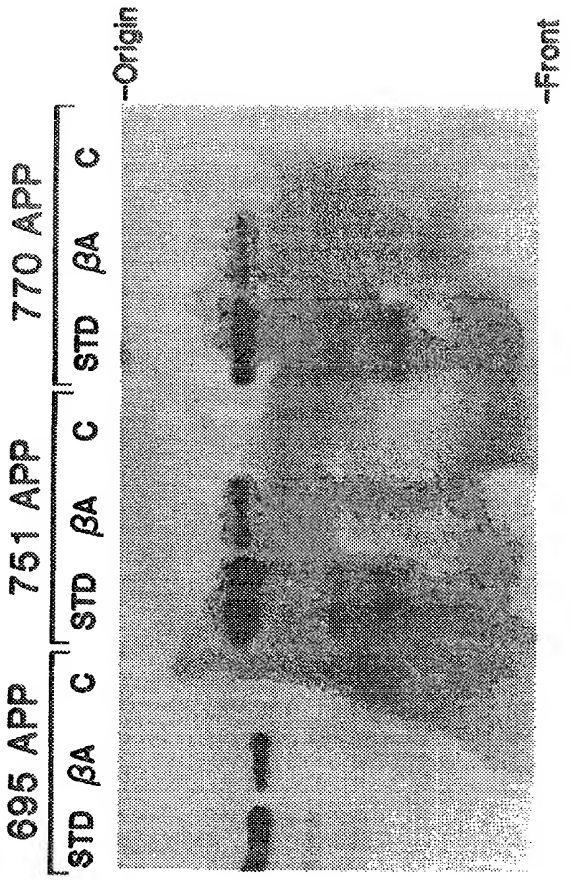
KUNITZ DOMAIN
19 AMINO ACID INSERT
AMYLOID

FIG.3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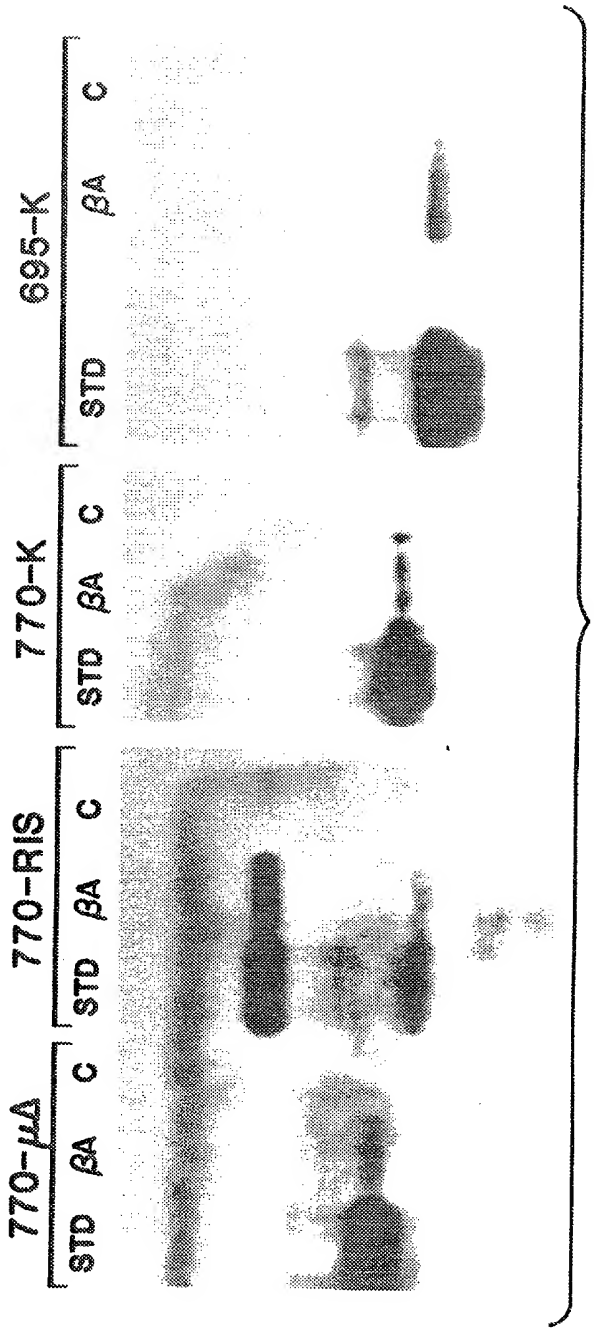
Origin
Front

FIG. 4A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770-K
695-K
STD
βA
C
STD
βA
C
STD
βA
C
STD
βA
C

FIG. 4B

FIG. 5A

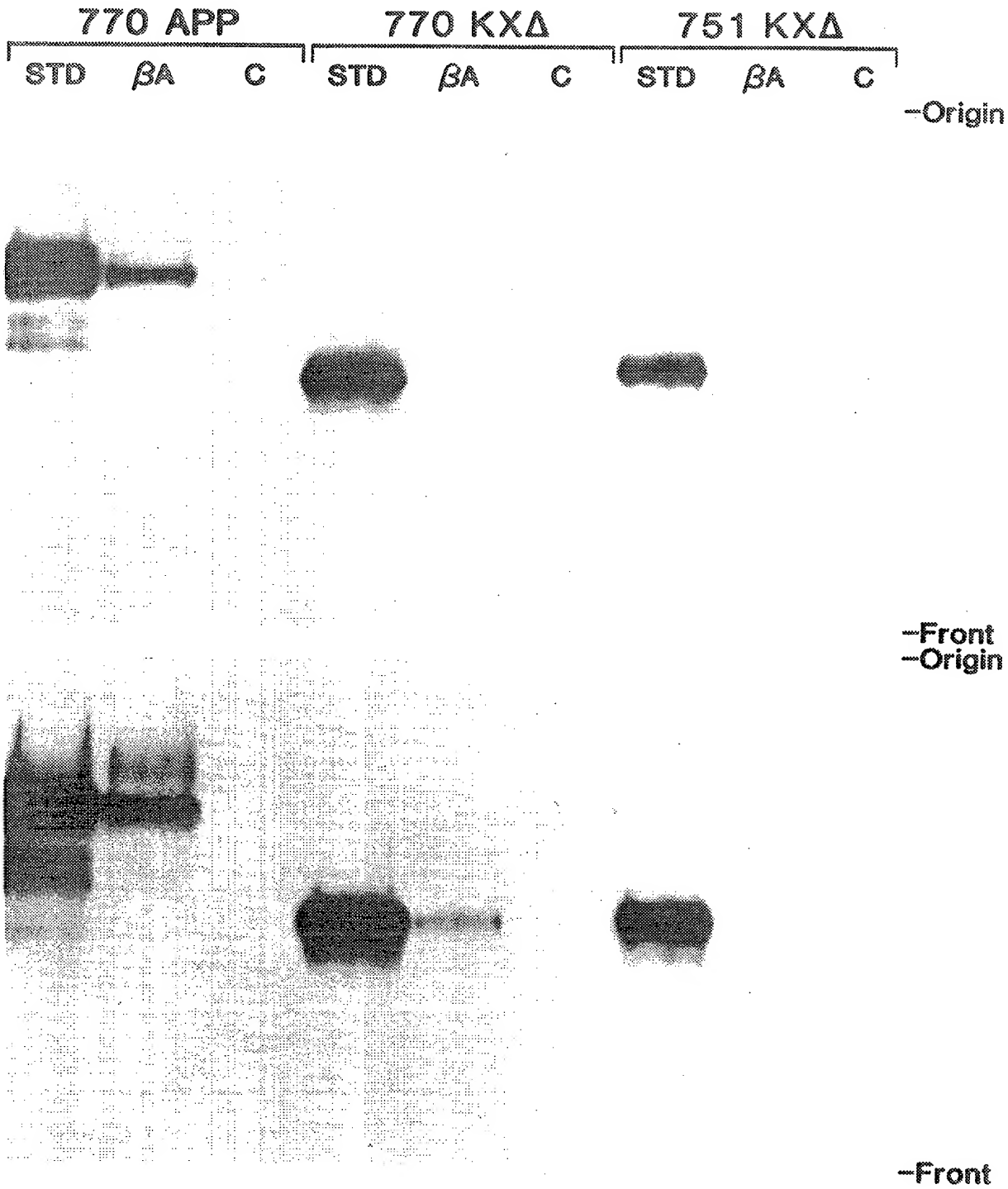
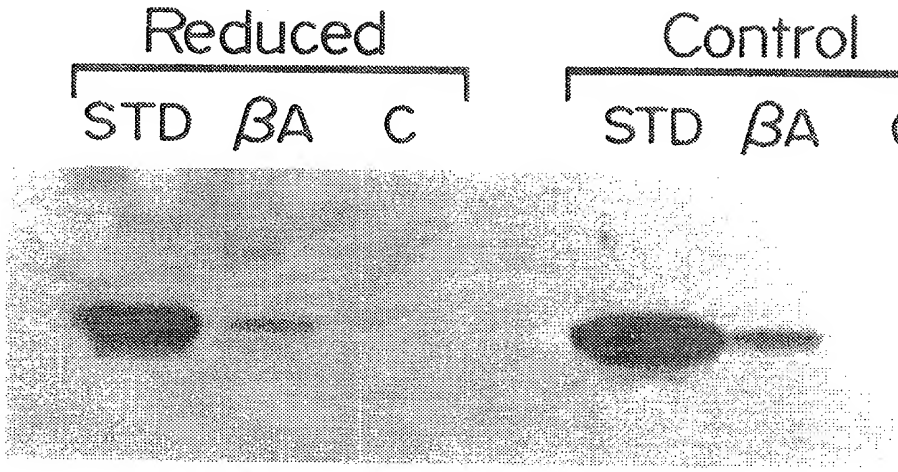

770 APP
770 KXΔ
751 KXΔ
STD βA C
STD βA C
STD βA C
Origin
Front
Origin
Front

FIG. 5B

FIG. 6A


Reduced
Control
STD βA C
STD βA C

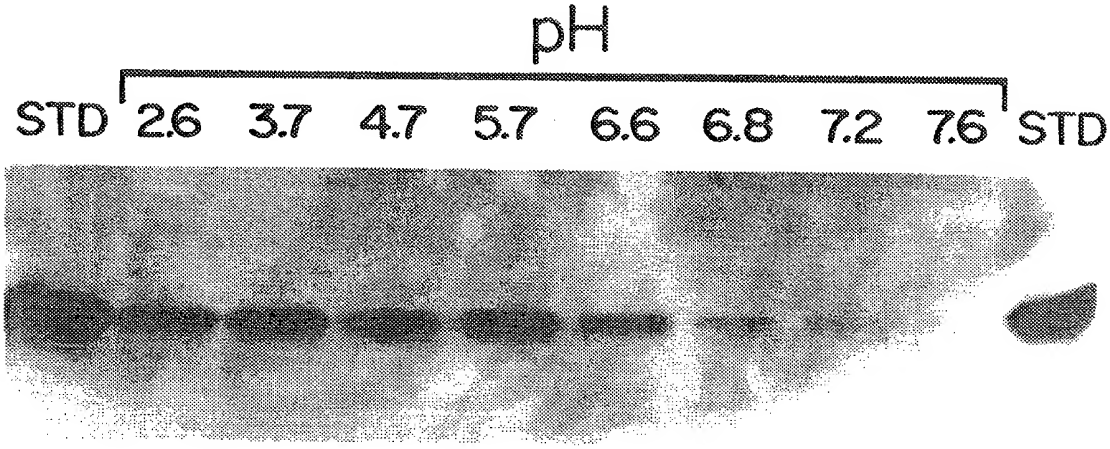
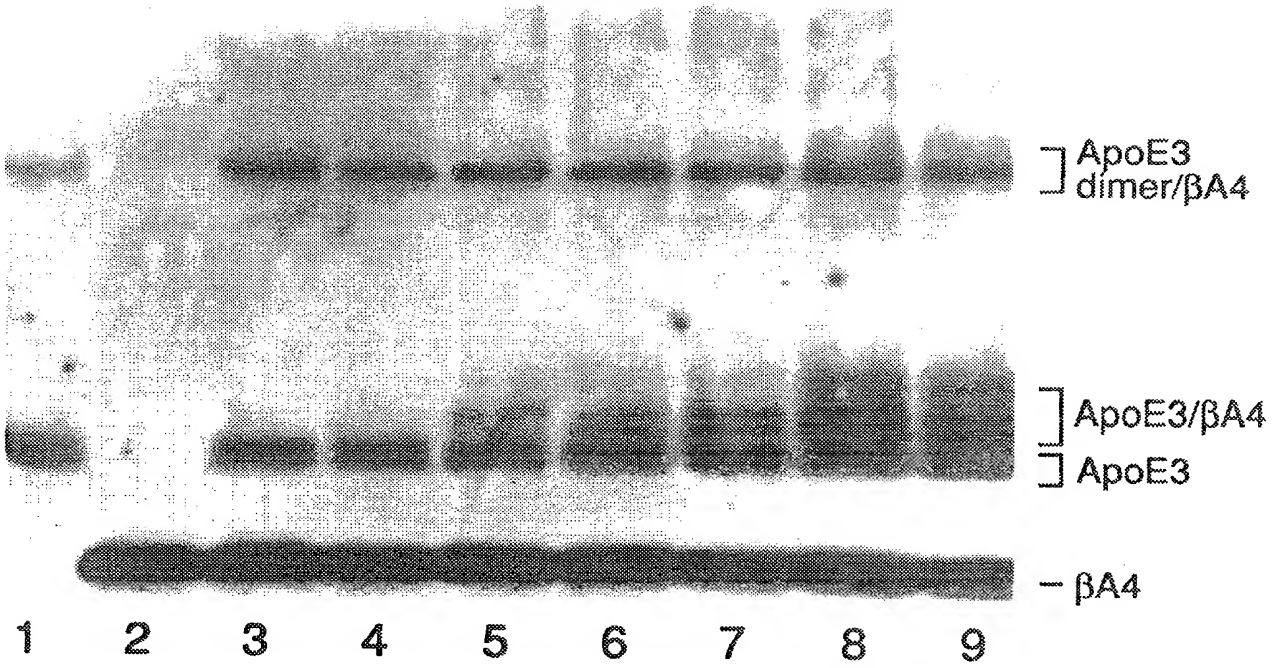

pH
STD 2.6 3.7 4.7 5.7 6.6 6.8 7.2 7.6 STD

FIG. 6B

FIG.7A


ApoE3 dimer/βA4
ApoE3/βA4
ApoE3
βA4
1 2 3 4 5 6 7 8 9

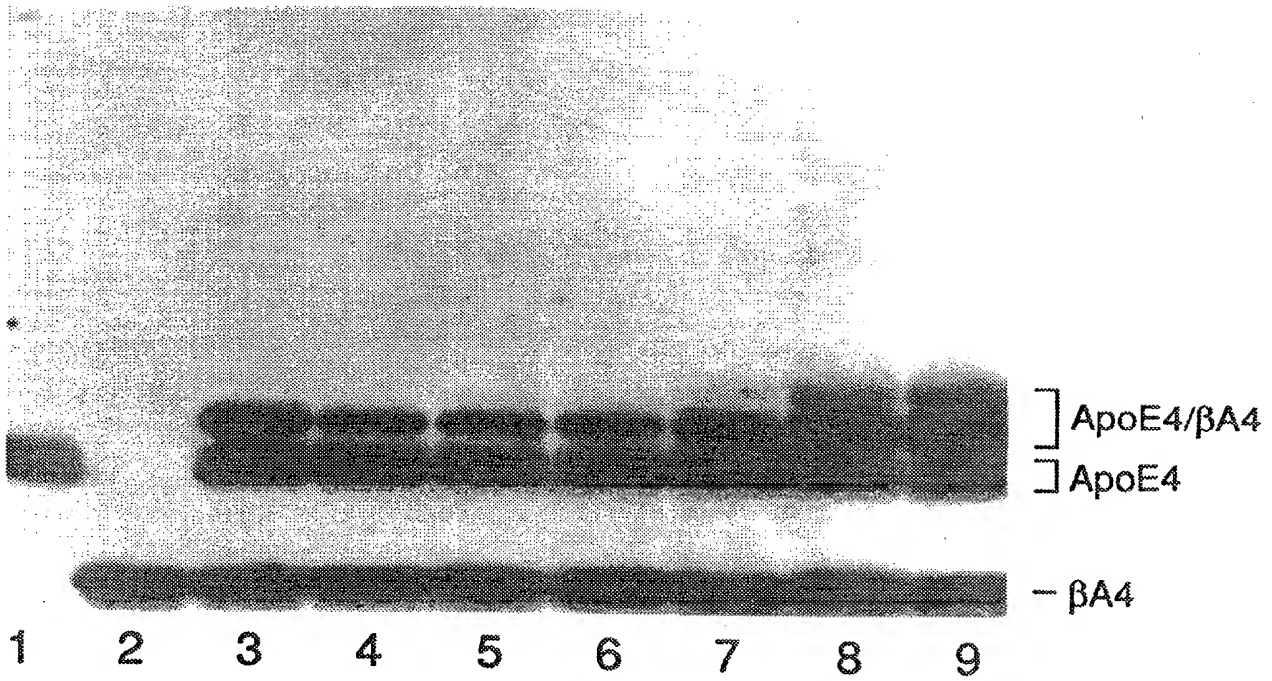
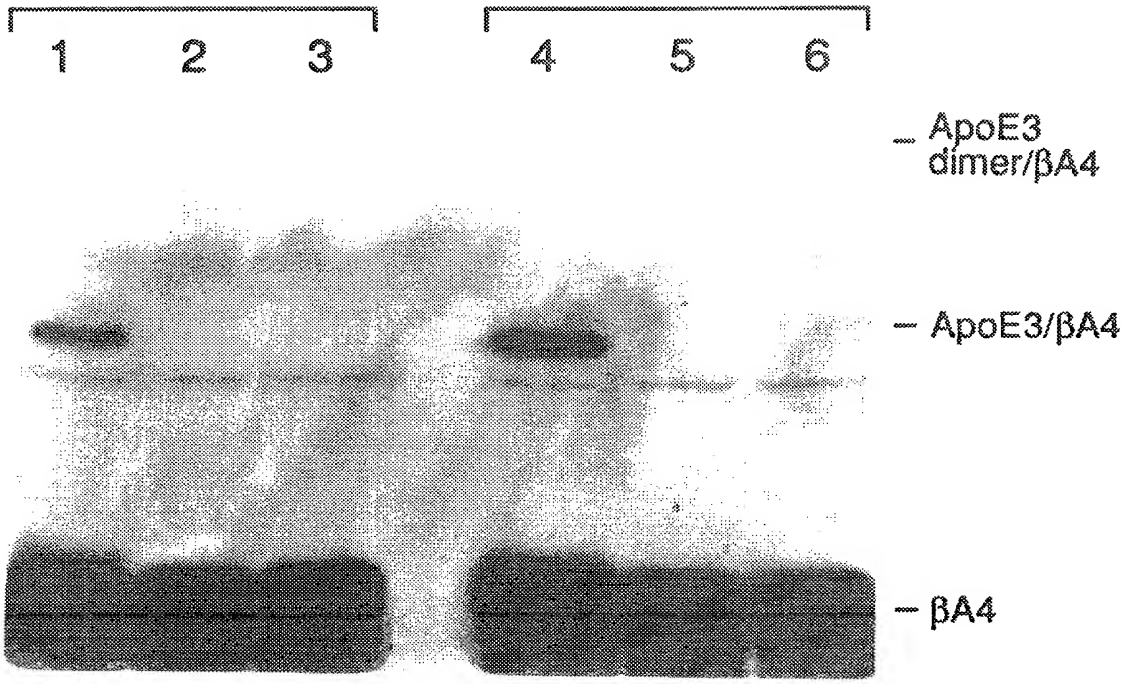

ApoE4/βA4
ApoE4
βA4
1 2 3 4 5 6 7 8 9

FIG.7B

FIG. 8A



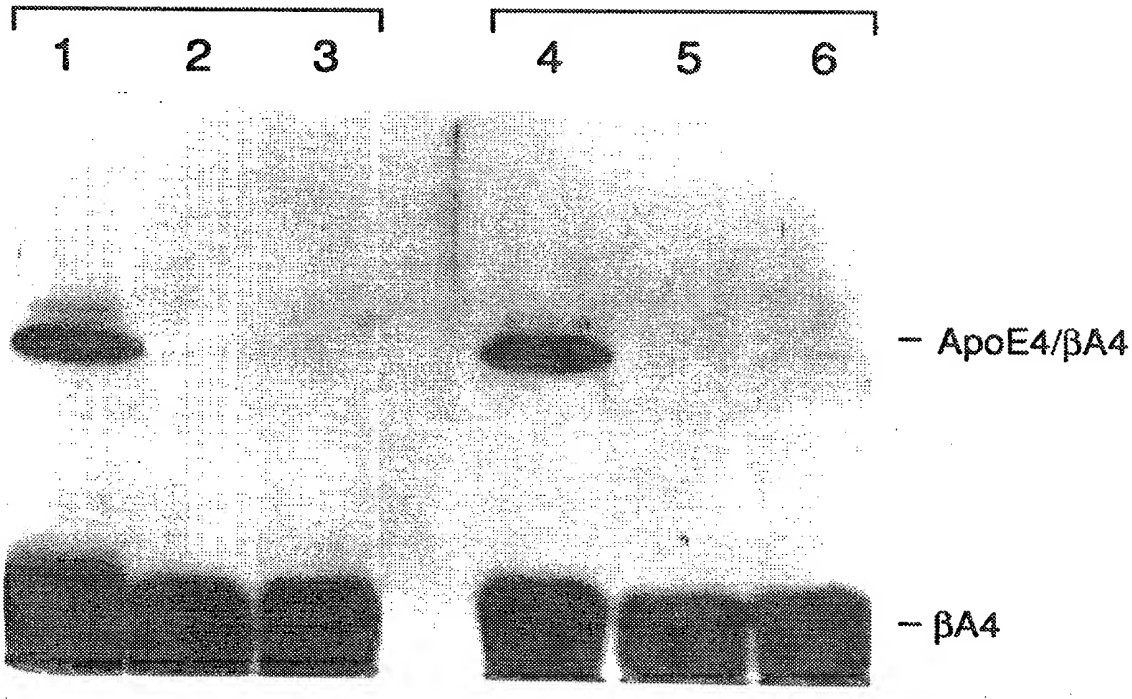


FIG. 8B

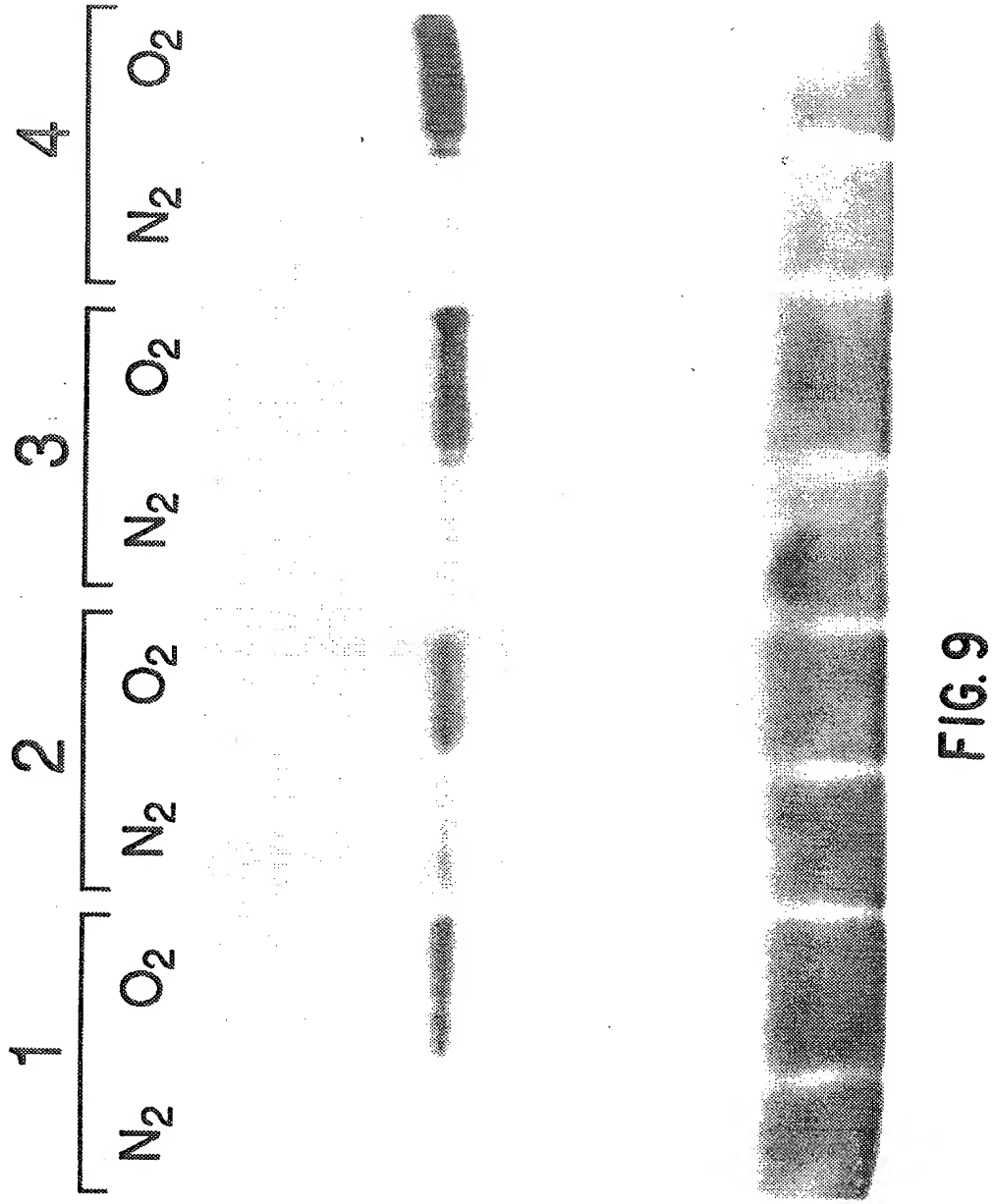


FIG. 9

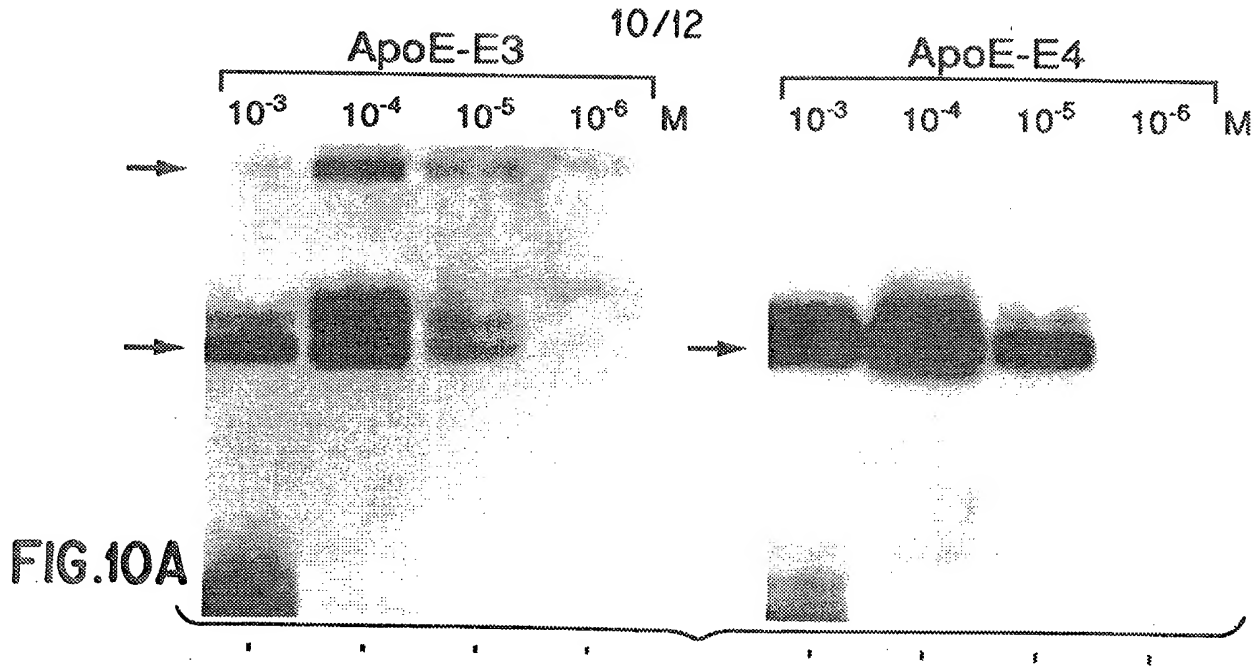


FIG.10A

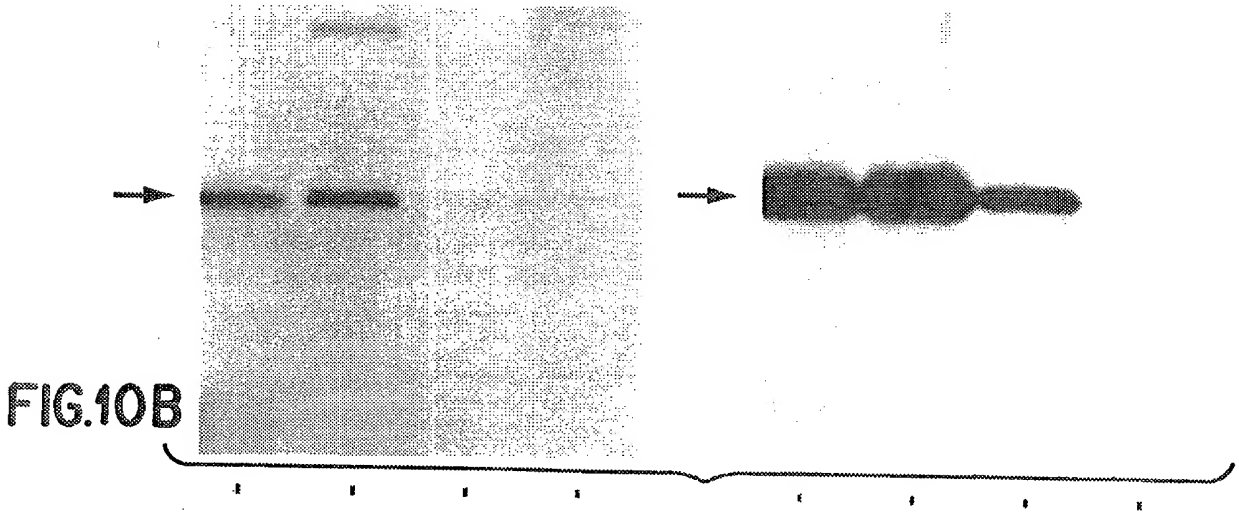


FIG.10B

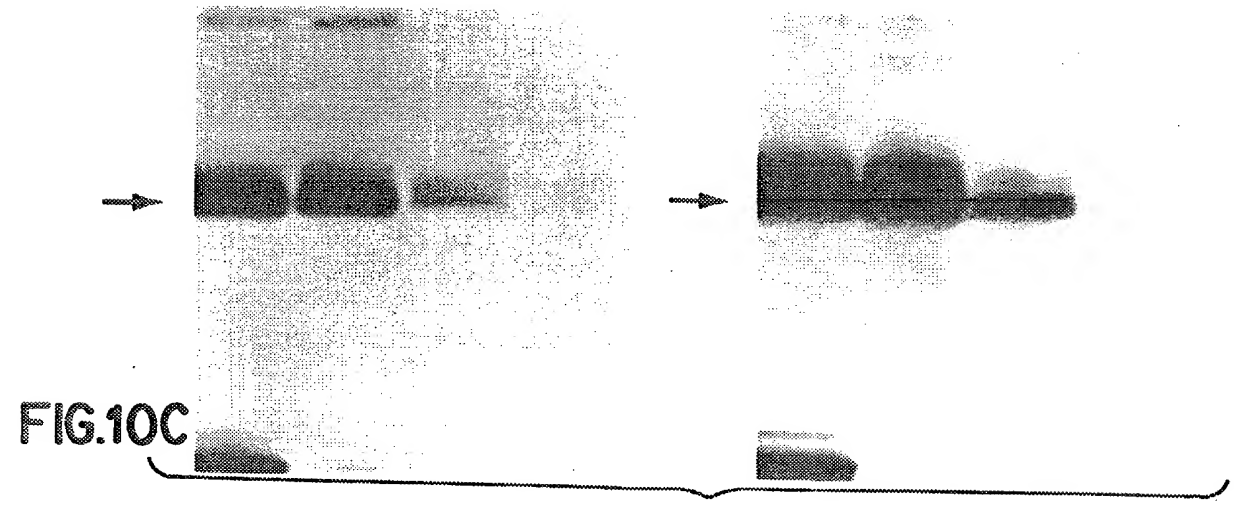


FIG.10C

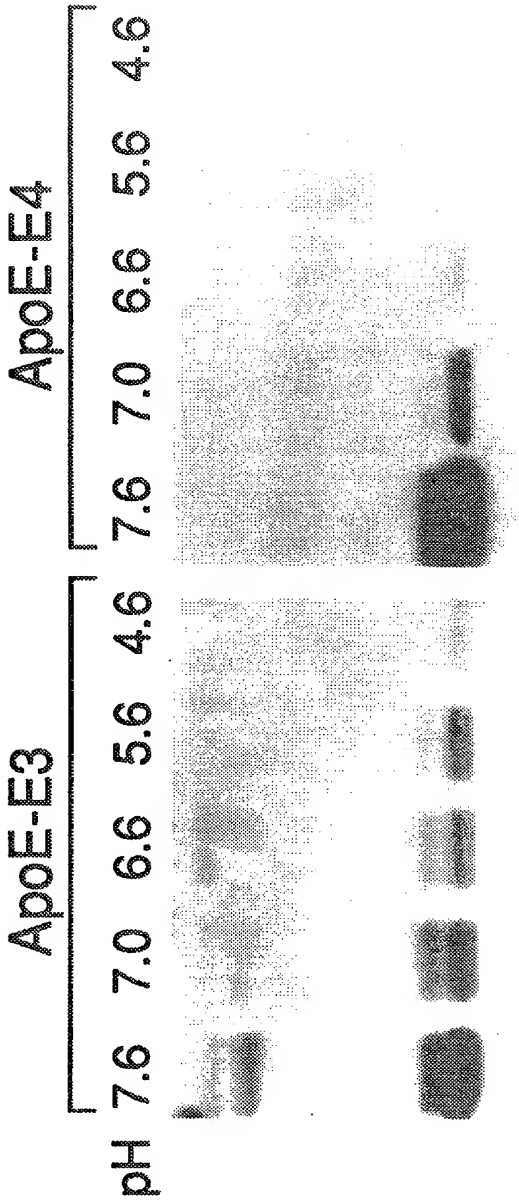


FIG. 11A

FIG. 11B

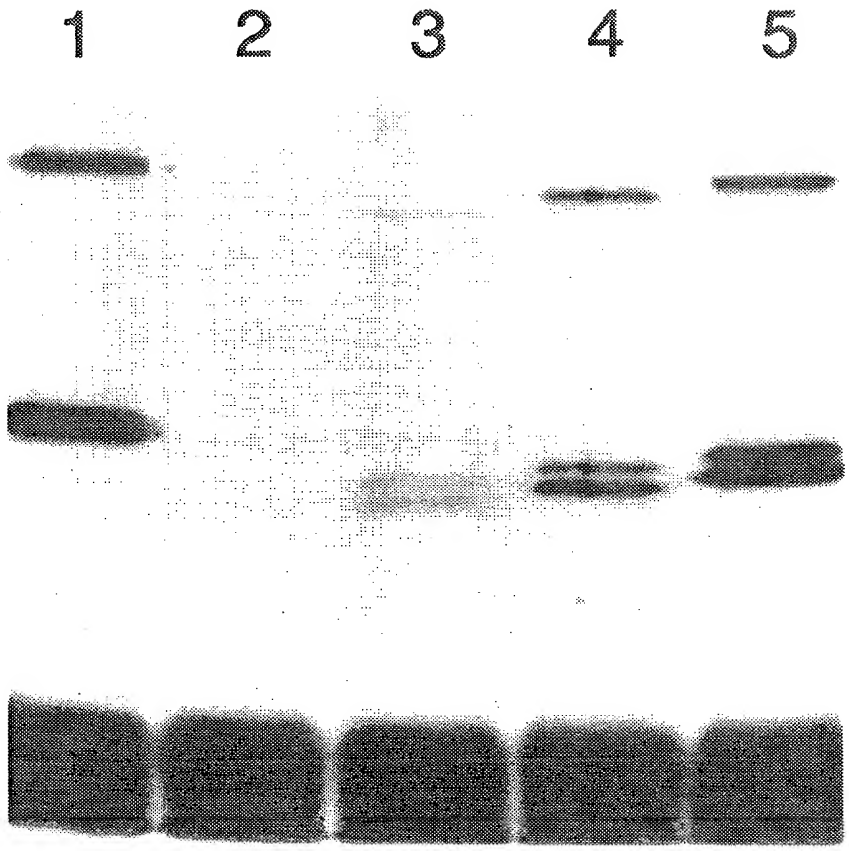


FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09772

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/53; 33/543
US CL :435/7.1; 436/518

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 7.9, 970; 436/518, 530, 531, 811; 422/50, 55, 56, 68.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE, EMBASE, DERWENT, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Biochemical and Biophysical Research Communications, Volume 141, No. 2, issued 15 December 1986, Castano et al, "In Vitro Formation of Amyloid Fibrils From Two Synthetic Peptides of Different Lengths Homologous to Alzheimer's Disease Beta-Protein", pages 782-789, especially see "Peptide Synthesis" on page 784.	1-6 10-21
Y	US, A, 4,923,901 (KOESTER ET AL) 08 May 1990, column 2, lines 10-31 and column 3, lines 26-45.	7-9

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 December 1993	Date of mailing of the international search report 13 JAN 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer CAROL E. BIDWELL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09772

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Science USA, Volume 86, issued August 1989, Palmert et al, "The Beta-Amyloid Protein Precursor of Alzheimer Disease Has Soluble Derivatives Found in Human Brain and Cerebrospinal Fluid", pages 6338-6342, especially see last complete paragraph on page 6341 and first complete paragraph on page 6342.	8-9
Y	WO, A, 92/03474 (POTTER) 05 March 1992, see entire document, especially page 12, lines 8-22 and claims 3 and 4.	10-37
Y,P	Proceedings of the National Academy of Science USA, Volume 90, issued March 1993, Strittmatter et al, "Apolipoprotein E: High-Avidity Binding to Beta-Amyloid and Increased Frequency of Type 4 Allele in Late-Onset Familial Alzheimer Disease", pages 1977-1981, especially see page 1977, second column first complete paragraph and page 1979, first complete paragraph.	22-37
Y,P	Proceedings of the National Academy of Science USA, Volume 90, issued September 1993, Strittmatter et al, "Binding of Human Apolipoprotein E to Synthetic Amyloid Peptide: Isoform-Specific Effects and Implications for Late-Onset Alzheimer Disease", pages 8098-8102, especially see Abstract on page 8098.	22-37
Y	The Journal of Biological Chemistry, Volume 267, No. 1, issued 05 January 1992, Burdick et al, "Assembly and Aggregation Properties of Synthetic Alzheimer's A4/Beta Amyloid Peptide Analogs", pages 546-554, especially see Abstract on page 546.	1-21
A,P	Science, Volume 261, issued 13 August 1993, Corder et al, "Gene Dose of Apolipoprotein E Type 4 Allele and the Risk of Alzheimer's Disease in Late Onset Families", pages 921-923	22-37
A,P	Science, Volume 261, issued 13 August 1993, Travis, "New Piece in Alzheimer's Puzzle", pages 828-829.	22-37